OPIOID MODULATION OF GONADOTROPIN RELEASING HORMONE RELEASE FROM THE HYPOTHALAMIC PREOPTIC AREA IN THE PIG

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ABSTRACT

Two experiments (Exp) were conducted to examine in vitro the release of gonadotropin releasing hormone (GnRH) from the hypothalamus after treatment with naloxone (NAL) or morphine (MOR). In Exp 1, hypothalamic-preoptic area (HYP-POA) collected from 3 market weight gilts at sacrifice and sagittally halved were perifused for 90 min prior to a 10 min pulse of morphine (MOR; 4.5×10^{-6} M) followed by NAL (3.1×10^{-5} M) during the last 5 min of MOR (MOR + NAL; n=3). The other half of the explants (n=3) were exposed to NAL for 5 min. Fragments were exposed to KCl (60 mM) at 175 min to assess residual GnRH releasability. In Exp 2, nine gilts were ovariectomized and received either oil vehicle im (V; n=3); 10 μg estradiol-17ß/kg BW im 42 hr before sacrifice (E; n=3); .85 mg progesterone/kg BW im twice daily for 6 d prior to sacrifice (P_4; n=3). Blood was collected to assess pituitary sensitivity to GnRH (.2 μg/kg BW) on the day prior to sacrifice. On the day of sacrifice HYP-POA explants were collected and treated as described in Exp 1 except tissue received only NAL. In Exp 1, NAL increased (P<.05) GnRH release. This response to NAL was attenuated (P<.05) by coadministration of MOR. Cumulative GnRH release after NAL was greater (P<.05) than after MOR + NAL. All tissues responded similarly to KCl with an increase (P<.05) in GnRH release. In Exp 2, pretreatment luteinizing hormone (LH) concentrations were lower (P<.05) in E gilts compared to V and P_4 animals with P_4 being lower (P<.05) than V gilts. LH response to GnRH was lower (P<.05) in E pigs than in V and P_4 animals, while the responses was similar between V and P_4 gilts. NAL increased GnRH release in all explants, whereas, KCl increased GnRH release in 6 of 9 explants. These results indicate that endogenous opioid peptides may modulate in vitro GnRH release from the hypothalamus in the gilt.

INTRODUCTION

Pulsatile luteinizing hormone (LH) secretion, elicited by episodic release of gonadotropin releasing hormone (GnRH) from the hypothalamus (1) subsequently regulates ovarian follicle development in the pig (2). We demonstrated that GnRH immunostained neurons are concentrated in the medial preoptic area, paraventricular nucleus, periventricular zone and medial basal hypothalamus (3). These neurons may interact with projections from proopiomelanocortin (POMC)- immunoreactive neurons located in the medial basal hypothalamus (4). Furthermore, in vitro (5) and in vivo (6-8) studies, using endogenous opioid peptide (EOP) agonists and antagonists, demonstrated that EOP modulate pulsatile LH secretion in the pig. Therefore, the objectives of the present study were two-fold; first, to determine if EOP modulate GnRH secretion from the hy-

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pothalamus in vitro, and secondly, if steroid milieu of the tissue donor influences EOP modulation of GnRH release.

MATERIALS AND METHODS

Tissue: Brain tissue was obtained from three market weight gilts (weighing 100-110 kg) after kosher slaughter. Animals were treated humanely and experimental procedures had prior approval by the USDA-ARS Animal Care Committee. The skull was opened and a block of tissue containing the entire hypothalamic-preoptic area (HYP-POA) was quickly excised. To isolate the HYP-POA from the block of brain tissue, the following cuts were made: rostral to the optic chiasm, rostral to the mammillary body, lateral to hypothalamic sulci and ventral to the anterior commissure. The resulting explants which did not contain the median eminence (ME) were sagittally halved and each half sliced into 0.8 mm sections. All pieces composing each half were placed into a flask containing oxygenated perifusion medium, brought to the laboratory, and immediately placed into perifusion micro-chambers with a 1.5 ml volume. In addition, at time of slaughter, ovaries were devoid of corpora albicantia and corpora lutea.

In vitro perifusion: Perifusion medium consisted of Delbecco's modified Eagle's medium and Ham's Nutrient Mixture F-12 (DME/F12; pH 7.3; 1.2 g $NaHCO_3$; .1% bovine serum albumin; .1 mM bacitracin; 5 mg amphotericin B; 5 mg kanamycin sulfate/l; Sigma Chemical Co., St. Louis, MO). The perifusion medium was oxygenated (95% O_2/5% CO_2), maintained at 37° C and pumped upward into the tissue chambers of an Acusyst-2 cell culture system (Endotronics, Coon Rapids, MN)at 100 µl/min. Effluent medium was collected continuously as 5-min fractions (500 µl). Fractions were collected into polypropylene vials on ice, acidified with 50 µl of 1N HCl, snap-frozen immediately and stored at -20° C. Samples were neutralized to pH 6-7 with 1N NaOH prior to radioimmunoassay (RIA) for GnRH.

After a 90 min stabilization period, half of the tissue fragments were exposed to morphine (MOR; 4.5×10^{-6} M) for 10 min and naloxone (NAL; 3.1×10^{-5} M, Sigma) during the last 5 min of MOR (MOR + NAL; n=3). Based on a preliminary study (data not shown), doses of NAL and MOR employed in the present study effectively stimulated or inhibited GnRH secretion, respectively. The other half of the tissue fragments were exposed to the same dose of NAL for 5 min as described above. All explants were exposed to 60 mM KCl for 5 min at 175 min to assess GnRH releasability. Naloxone, MOR and KCl were diluted in perifusion medium.

Experiment II: Nine gilts, weighing 116 ± 2 kg and which had displayed one or more estrous cycles of 18-22 d, were ovariectomized (OVX). All gilts were housed in individual pens in an environmentally controlled building and exposed to a constant ambient temperature of 22° C and an artificial photoperiod of 12:12 hr light:dark. Pigs were fed between 0700 and 0800 hr a corn-soybean meal ration (14% crude protein) supplemented with vitamins and minerals, according to National Research Council (9) guidelines. Starting approximately 2 weeks after OVX, the experiment was conducted with the following treatments: corn oil vehicle im (V; n=3); 10 µg estradiol-17ß/kg BW im 42 hr before sacrifice (E; n=3); .85 mg progesterone/kg BW im twice daily for six d prior to sacrifice (P_4; n=3). Gilts in the V and E groups also received corn oil injection twice daily for six d prior to sacrifice. Progesterone treatment was employed in order to simulate luteal phase serum concentrations of P_4 (6) while the dose of E was used to simulate late follicular phase serum concentrations of E (6). Two d prior to sacrifice, an indwelling catheter was placed into the jugular vein of each pig (10). Blood samples were collected the following day every 15 min for 2 hr prior to and 1 hr after GnRH (.2

μg/kg BW). Based on previous observations, doses of GnRH employed in the present study were optimal for assessing differences in pituitary sensitivity (11). Blood samples were allowed to clot at 4° C for 24 hr and serum was harvested after centrifugation and stored at -20° C. On the day of sacrifice, tissue was collected, perifused and treated as described in Exp 1, except tissues were exposed only to a 5 min pulse of NAL (3.1 × 10^{-5} M) starting at 85 min followed by a 5 min pulse of 60 mM KCl starting 175 min.

Hormone Assays: *GnRH RIA*. Procedures for measurement of GnRH were similar to those described by Sesti and Britt (12), except the anti-GnRH antiserum RDK-123 used in this assay was raised in our laboratory by immunizing pigs against native GnRH conjugated to BSA as described by Esbenshade and Britt (13). The antiserum diluted at 1:10,000 bound 30% of added ^{125}I-labelled GnRH. RDK-123 does not crossreact (<.001%) with LH, FSH, ACTH, prolactin, GRF, CRF, TRH, salmon GnRH and ß-endorphin, somatostatin (<.01%), or the GnRH agonists (<.1%), des-Gly 10- GnRH and Gly-OH-GnRH. The antiserum is directed against the carboxy-terminal of the native GnRH molecule since GnRH fragments lacking the first two or three n-terminal amino acids showed 100% crossreactivity. Sensitivity of the assay was .2 pg/tube at 95% binding. Increasing dilutions of homogenates of porcine median eminence and perifusion effluent produced displacement curves that were parallel (P>.1) to that produced by increasing amounts of standard GnRH (Sigma). Recovery of GnRH (1 to 35 pg) added to perifusion effluent was 107 ± 3%. Samples were assayed in duplicate 100 μl volumes and data expressed as pg/5 min fraction. Intra- and inter-assay CV were 6.1 and 7.7%, respectively.

Serum concentrations of LH (14) were quantified by RIA on all samples. Sensitivity of the assay was .15 ng/ml of LH. Intra-assay and interassay coefficients of variation were 4.8% and 9.0%, respectively. Serum P_4 (15) and E (16) were quantified by RIA on the first sample collected from each gilt. Sensitivity of the P_4 and E assays were .5 ng/ml and .6 pg/ml, respectively. Intra-assay and interassay co-efficients of variation were 9.3% and 15.0% for P_4 and 13.65 and 19.1% for E, respectively.

Statistical analysis: Due to variability in GnRH secretion (washout period), fractions collected during the first 50 min were discarded. For each tissue fragment, basal GnRH concentrations, number of GnRH pulses, and GnRH peak magnitude were determined by Pulsar analysis using a 5% criterion of variation (17). Peak response was the maximal response above baseline mean after treatment. Temporal analysis of the perifusion system revealed that 10 min was required from initiation of treatment until the agent reached the tissue chambers and an additional 5 min until the agent reached the collection tube. Therefore, peak responses of GnRH release were not expected before the third or fourth fraction after initiation of treatment. Cumulative GnRH secretion was calculated as the mean of fractions collected for 70 min after NAL or MOR + NAL treatment.

Differences in basal secretion of GnRH, peak GnRH response to NAL, MOR + NAL or KCl and cumulative GnRH secretion were determined by analysis of variance using the general linear model procedure of the Statistical Analysis System (SAS;18).

To determine the effect of steroid treatment on serum LH concentrations and LH response to GnRH, data were divided into two periods for each pig. Period one represented the mean of samples collected prior to GnRH treatment and Period 2 represented the mean of samples collected after the GnRH challenge. Period means were then subjected to the general linear model split plot- in-time analysis of variance procedure of SAS (18). The statistical model included treatment, period and treatment x period interaction. Effects of treatment were tested using animal within treatment as the error term. Treatment differences were determined by least-squares contrasts (18).

RESULTS

Experiment I: Basal GnRH secretion was similar for NAL and MOR + NAL explants (Table 1). Exposure of the HYP-POA explants to NAL alone increased (P<.05) GnRH secretion. However, this response was antagonized (P<.05) in the presence of MOR (Figure 1). Cumulative GnRH secretion during the 70-min period following NAL was greater (P<.05) than following MOR + NAL (Table 1). All tissues responded similarly to K^+ with an increase (P<.05) in GnRH release (11.0 ± 1.3 pg/fraction; Figure 1).

Experiment II: Serum E concentrations were nondetectable in V and P_4 gilts, but averaged 24 ± 7 pg/ml for E gilts. Serum P_4 was nondetectable in V and E animals, but averaged 22 ± 5 ng/ml in P_4 gilts. Pretreatment serum LH concentrations were lower (P<.05) in E gilts compared to V and P_4 animals with P_4 being lower (P<.05) than V gilts (Figure 2). The LH response to GnRH was lower (P<.05) in E pigs than in V and P_4 animals, while the response was similar among V and P_4 gilts (Figure 2).

Profiles of GnRH release from HYP-POA explants collected from V, P_4 and E animals are presented in Figure 3. Basal GnRH secretion was similar for all groups (Table

TABLE 1. THE EFFECT OF MORPHINE (MOR) PLUS NALOXONE (MOR + NAL), OR NAL ALONE ON GnRH RELEASE FROM THE HYP-POA TISSUE EXPLANTS.[a]

Treatment	No.	Basal GnRH[b]	Peak GnRH after M+N or N[b]	Cumulative GnRH after M+N or N[c] (pg)
NAL	3	8.1 ± 1.8	16.2 ± .6[d]*	149.8 ± 22.5[d]
MOR + NAL	3	5.9 ± 1.0	10.2 ± .7[e]*	95.3 ± 22.5[e]

[a]mean ± SE. [b]pg/fraction. [c]Cumulative GnRH secretion for 70 min after MOR + NAL or NAL.
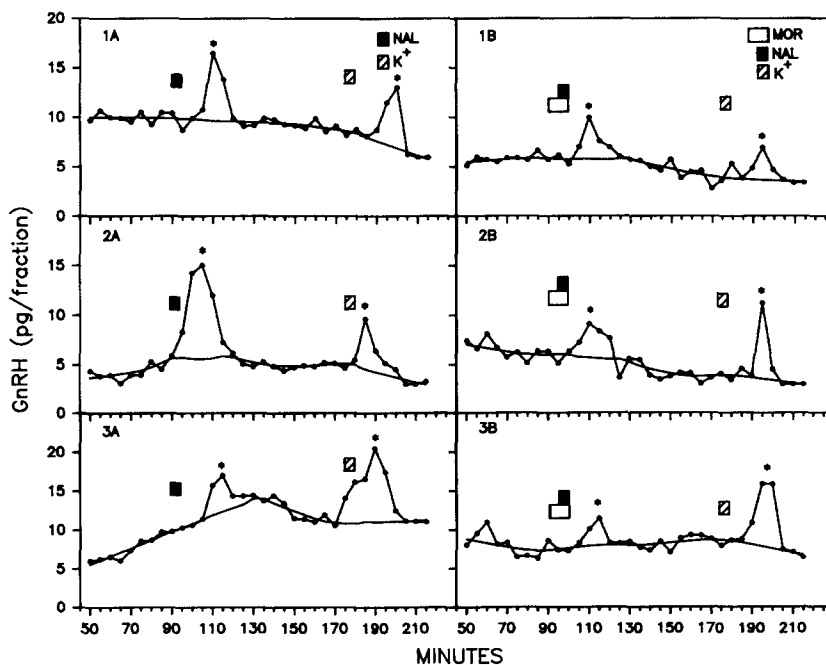Means in a column with different superscripts differ.[d,e] (P<.05). Means in a row differ from basal GnRH. *(P<.05).

Figure 1. Effect of naloxone (NAL; 3.1×10^{-5} M) alone, morphine (MOR; 4.5×10^{-6} M) plus NAL (MOR + NAL) and K^+ (60 mM) on gonadotropin releasing hormone (GnRH) release from paired hypothalamic-preoptic area halves. Basal GnRH release estimated by Pulsar analysis is shown as smooth lines. Each asterisk denotes an increase (P<.05) in GnRH concentrations in a 5 min fraction.
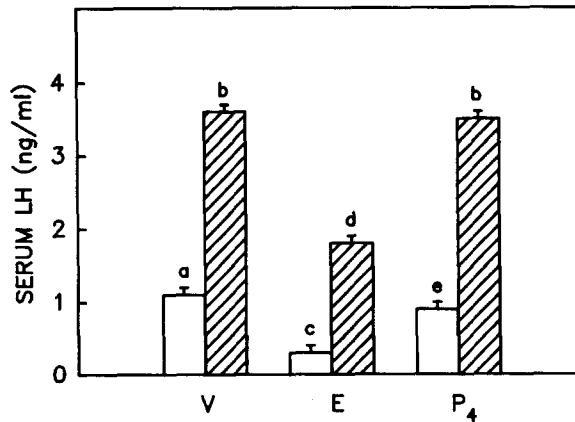
Figure 2. Pretreatment serum LH concentrations (open bars) and mean serum luteinizing hormone (LH) concentrations (striped bars) after gonadotropin releasing hormone (GnRH) for vehicle (V; n=3)- , estradiol (E; n=3)- and progesterone (P_4; n=3)-treated gilts. Values are means ± SE. Bars with different letters differ (P<.05).
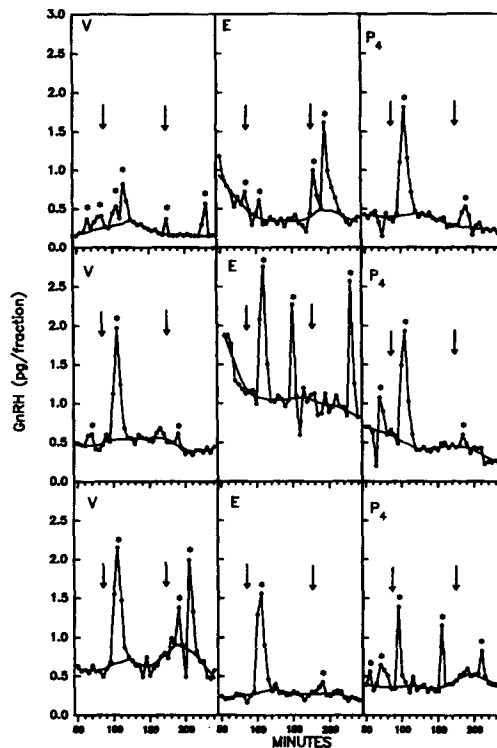

Figure 3. Effect of naloxone (NAL; 3.1 x 10^{-5} M) and K^+ (60 mM) on gonadotropin releasing hormone (GnRH) release from hypothalamic-preoptic area halves from vehicle (V)-, estradiol (E)- and progesterone (P_4)-treated gilts. Basal GnRH release estimated by Pulsar analysis is shown as smooth lines. Arrows indicated initiation of NAL (time = 85 min) and K^+ (time = 175 min) treatments. Each asterisk denotes an increase (P<.05) in GnRH concentration in a 5 min fraction.

2). Exposure of the HYP-POA explants to NAL increased (P<.05) GnRH release in all explants. Magnitude of the GnRH response to NAL was similar among all groups (Table 2). All but three tissues responded to K^+ with increased (P<.05) GnRH release (Figure 3). However, these tissues were considered viable, since they continued to exhibit GnRH pulses (Figure 3).

TABLE 2. THE EFFECT OF NALOXONE (NAL) AND K$^+$ ON GnRH RELEASE FROM THE HYP-POA TISSUE
EXPLANTS FROM VEHICLE (V), ESTRADIOL (E) OR PROGESTERONE (P$_4$) TREATED OVX GILTS.[a]

Treatment	No.	Basal GnRH[b]	Peak GnRH after NAL[b]
V	3	.5 ± .1	1.6 ± .5[*]
E	3	.6 ± .3	1.6 ± .6[*]
P$_4$	3	.4 ± .1	1.7 ± .2[*]

[a]Mean ± SE
[b]pg/fraction
Means in a row differ from basal GnRH [*](P<.05)

DISCUSSION

Results from the present study demonstrate that neurons present in the HYP-POA tissue are an important site for EOP modulation of GnRH release in the pig. This is in agreement with previous in vitro studies using medial basal hypothalamus (MBH) explants of rats and humans or median eminence of cattle (19), which demonstrated that NAL stimulated GnRH release (20-22). Measurements of NAL-induced secretion of GnRH in pituitary portal blood of rats (23), sheep (24) and bull calves (25) are consistent with the in vitro studies. Moreover, Rasmussen et al. (26) demonstrated that the isolated human MBH in vitro released GnRH in discrete spontaneous pulses. However, there was no evidence of endogenous rhythmic pulses of GnRH secretion in the present study, although some spontaneous pulses of GnRH were detected in Exp 2.

Anatomical studies of rat POA (27) and primate MBH (28) revealed EOP synapses on GnRH neurons, indicating possible direct influences of EOP on GnRH neuronal activity. We demonstrated that POMC perikarya and immunoreactive fibers are well positioned to interact directly with GnRH neurons within the hypothalamus of the pig (4). Alternatively, pharmacological evidence in the pig suggests that EOP also modulate GnRH neuronal activity indirectly, via noradrenergic neurons (29). In the rat, NAL stimulated both GnRH and catecholamine release from MBH, MBH-POA and ME fragments in vitro (30,31), while MOR treatment blocked electrically-induced release of norepinephrine from POA fragments (32).

Failure of MOR to completely block the present NAL-induced release of GnRH may, in part, be related to dose and/or time of exposure of the tissue to MOR. Cumulative GnRH secretion after NAL was greater than after MOR + NAL, suggesting that MOR antagonized NAL-induced GnRH release. GnRH releasability and presumably tissue viability was confirmed following a significant increase in GnRH concentrations after KCl challenge in all tissues.

In Experiment II, NAL stimulated GnRH release without regard to steroid milieu of the tissue donor. In contrast, in vivo administration of NAL stimulated LH secretion during the luteal phase but not during the follicular phase of the estrous cycle or in OVX mature or prepuberal gilts (6,16). A similar paradigm was reported for the aging female rat, in which NAL-induced release of LH declined with age (33), while the ability of NAL to evoke GnRH release from hypothalamic fragments was similar for young and aged rats (34). There are several possible explanations for this paradox: first, differences in the ability of in vivo injections of NAL to reach relevent tissues involved in modulating GnRH release; second, alterations in other neuronal systems which influence pituitary responsiveness to GnRH in vivo, e.g., LH response to GnRH was attenuated in E-treated pigs in the present study; third, NAL may more effectively stimulate GnRH release from tissue in vitro without regard to steroid milieu of the tissue donor than in vivo, because excitatory and/or inhibitory input on GnRH neurons that originate in areas outside of the HYP-POA, which may be potential sites for steroid action, were excluded due to tissue dissection; and last, differences in response between in vivo and

in vitro may be associated with frequency and amplitude of episodic pulses of GnRH as opposed to the releasable pool of GnRH from the HYP-POA.

The ME is the physiological site of GnRH secretion that directly modulates pituitary LH secretion. In the present study, opioid inhibition within HYP-POA, which was devoid of ME, might occur at GnRH perikarya and dentrites to suppress GnRH neuronal activity preventing GnRH release from axon terminals in the ME. In addition, NAL-induced GnRH release from HYP-POA explants could also reflect opioid suppression of GnRH activity unrelated to gonadotropin secretion. Similar results have been reported for the bovine (19). Moreover, the ability of NAL to stimulate GnRH release from HYP-POA sections suggests an autonomous EOP systems. Indeed, Kineman et al. (4) demonstrated that the POMC-containing system in the forebrain of the pig originates in the arcuate area with projections to hypothalamic and extrahypothalamic structures, indicating, anatomically, the POMC system is well positioned to interact directly with GnRH-containing neurons (4).

Therefore, we conclude that neurons in HYP-POA are sites for EOP regulation of GnRH secretion in the pig. However, further studies will be required to define the influence of reproductive state on EOP modulation of GnRH release.

ACKNOWLEDGMENTS/FOOTNOTES

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